Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8919–8923, December 1988
Biochemistry

Phosphorylation of nitrogen regulator I (NR_I) of *Escherichia coli*

(histidine phosphate/aspartate phosphate/phosphate transferase)

VERENA WEISS AND BORIS MAGASANIK*

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by Boris Magasanik, August 26, 1988

ABSTRACT **It has previously been shown that phosphorylated nitrogen regulator I (NR_I-phosphate) is the activator responsible for increasing the transcription of *glnA*, the structural gene for glutamine synthetase, and that NR_{II} catalyzes the transfer of the γ-phosphate of ATP to NR_I. We have now shown that the reaction of ATP with NR_{II} results in the reversible transfer of the γ-phosphate of ATP to a histidine residue of NR_{II}. In turn, NR_{II}-phosphate transfers its phosphate reversibly to an aspartic residue of NR_I. NR_I-phosphate is hydrolyzed to NR_I and inorganic phosphate in a divalent cation-requiring autocatalytic reaction.**

Earlier studies from this laboratory have shown that the initiation of transcription at *glnAp2*, the nitrogen-regulated promoter of *glnA*, the structural gene for glutamine synthetase of *Escherichia coli*, requires, in addition to σ^{54} RNA polymerase (RNA polymerase with a 54-kDa σ subunit), the regulatory proteins NR_I and NR_{II} (1). The role of NR_{II} was shown to be the catalysis of the transfer of the γ-phosphate of ATP to NR_I (2). NR_I-phosphate in turn brings about the isomerization of the closed σ^{54} RNA polymerase–*glnAp2* complex to the open complex (3). We describe now the mechanism responsible for the phosphorylation of NR_I.

MATERIALS AND METHODS

Chemicals and Reagents. Homoserine, homoserine lactone, and sodium borohydride were obtained from Serva (New York); histidine was obtained from Eastman Kodak; Whatman 3 MM paper, nitrocellulose membranes, and glass fiber filters were from Schleicher & Schuell; silica plates and polyethyleneimine-cellulose sheets were obtained from Baker; [γ-^{32}P]ATP, [α-^{32}P]ATP, and sodium boro[^{3}H]hydride were obtained from Amersham.

τ-Phosphohistidine (histidine 3-phosphate) was synthesized by the reaction of histidine with the potassium salt of phosphoramidate (4, 5). The latter was synthesized according to Sheridan *et al.* (6).

Purified Proteins. The source of NR_I was a 20-liter culture of *E. coli* strain TH19/pTH806 grown at 37°C as previously described (1). Sonication, streptomycin sulfate precipitation, and ammonium sulfate precipitation were done as previously described (7). The protein was further purified on a heparin-Sepharose column (Pharmacia) in Tris·HCl buffer with a linear NaCl gradient (unpublished results). NR_{II} was purified as previously described (8).

The concentrations of NR_I and NR_{II} were estimated from their absorbances at 280 nm, using $A^{1\%} = 9.1$ for NR_I and $A^{1\%} = 4.15$ for NR_{II}, as calculated from their amino acid compositions and expressed in terms of the monomer.

Isolation of NR_I-Phosphate. In a typical experiment, NR_I (3 μM) was incubated in 65 mM Tris·HCl/120 mM NaCl/5 mM $MgCl_2$/0.5 mM EDTA/10% (vol/vol) glycerol, pH 8, with 0.4 mM [γ-^{32}P]ATP (1–1.5 Ci/mmol; 1 Ci = 37 GBq) and 2.8 μM NR_{II} at 37°C for 30 min (final volume 200 μl). The mixture was chilled, added to 70 μl of heparin-Sepharose (equilibrated in 50 mM Tris·HCl, pH 8), mixed, and centrifuged. The supernatant was discarded and the residue was washed in the cold twice with 1.4 ml of 50 mM Tris·HCl, pH 8, and six times with 50 mM Tris·HCl/1 mM EDTA, pH 8. The NR_I was eluted three times with 150 μl of 50 mM Tris·HCl/1 M NaCl, pH 8. The eluted NR_I was usually 10% phosphorylated.

For sodium borohydride reduction, NR_I was phosphorylated in a final volume of 1.25 ml and loaded on a 1.5-ml heparin-Sepharose column equilibrated in 50 mM Tris·HCl, pH 8, washed with 7 ml of 50 mM Tris·HCl/1 mM EDTA, pH 8, and eluted with 10 ml of 50 mM Tris·HCl/1 mM EDTA/1 M NaCl. The fractions containing phosphorylated NR_I (3 ml) were pooled. Phosphorylated NR_I was precipitated on ice with 12 ml of 5% trichloroacetic acid, washed with 12 ml of ethanol/diethyl ether (1:1, vol/vol), and evaporated to dryness.

Isolation of NR_{II}-Phosphate. NR_{II} (7 μM) in 200 μl of 75 mM Tris·HCl/150 mM NaCl/5 mM $MgCl_2$/0.5 mM EDTA/20% (vol/vol) glycerol, pH 8, was treated with 0.4 mM [γ-^{32}P]ATP (1 Ci/mmol) for 15 min at 37°C. The protein was precipitated at 0°C for 15 min by the addition of 400 μl of an ammonium sulfate solution saturated at room temperature and was collected by centrifugation. The precipitate was dissolved in 100 μl of 150 mM Tris·HCl/1 mM EDTA, pH 8, and desalted by passage through a 10-ml Sephadex G-25 column.

Identification of Acyl Phosphates by Borohydride Reduction. The procedure described by Degani and Boyer (14) and Nishigaki *et al.* (9) was used with minor modifications as described in the legend to Fig. 7.

Identification of Phosphohistidine in NR_{II}-Phosphate. This was done as described by Smith *et al.* (10) with minor modifications as described in the legend to Fig. 4. Phosphohistidine was detected after two-dimensional chromatography by spraying the plates with 0.1 M HCl and heating at 100°C for 15 min, followed by treatment with the Pauly diazo reagent. $NaDodSO_4$/PAGE was done as described by Laemmli (11).

RESULTS

Phosphorylation of NR_{II}. The incubation of NR_{II} with ATP resulted in the transfer of the γ-phosphate of ATP to NR_{II}. In the experiments illustrated in Fig. 1, reaction mixtures containing NR_{II} and ATP labeled with ^{32}P in the γ position were spotted on nitrocellulose paper filters, which were then washed with Tris·HCl buffer to remove the nucleotides. Incorporation of radioactivity occurred only when ATP was labeled in the γ position (Fig. 1), not when it was labeled in the α position (data not shown). The transfer of the phosphate to NR_{II}, which required a divalent cation such as Mg^{2+}, proceeded rapidly at 37°C and more slowly at 0°C. The incorporation of phosphate reached a maximum of 0.35 mol of phosphate per mol of NR_{II} monomer.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NR_I and NR_{II}, nitrogen regulators I and II.

*To whom reprint requests should be addressed.

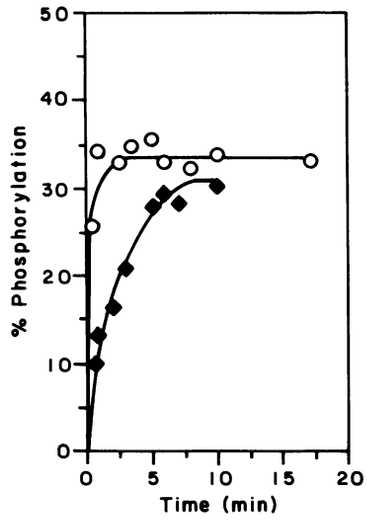

FIG. 1. Time course of incorporation of ^{32}P into NR_{II}. NR_{II} (3.5 μM) and bovine serum albumin as a carrier (50 μg/ml) was incubated in 50 μl of 60 mM Tris·HCl/75 mM NaCl/5 mM $MgCl_2$/0.3 mM EDTA/10% glycerol, pH 8, with 0.4 mM [γ-^{32}P]ATP (500 Ci/mol) at 37°C (○) and 0°C (♦). At the indicated time 5-μl samples were spotted on nitrocellulose paper and washed with 45 ml of 50 mM Tris·HCl buffer, pH 8, and their radioactivities were measured in a Beckman liquid scintillation counter.

Transfer of Phosphate from NR_{II}-Phosphate to NR_I or ADP. We were able to isolate NR_{II}-phosphate free of ATP by precipitation with ammonium sulfate followed by gel filtration chromatography. The results illustrated in Fig. 2*A* show that the incubation of NR_{II}-[^{32}P]phosphate with NR_I resulted in the transfer of the radioactive phosphate to NR_I. This transfer proceeded slowly in the absence of Mg^{2+} and rapidly in its presence; in the latter case most of the radioactivity had disappeared from NR_{II} and was found in NR_I after 5 min. Incubation of NR_{II}-[^{32}P]phosphate with ADP and Mg^{2+} resulted in the disappearance of radioactivity from NR_{II} (Fig. 2*A*) and in its appearance in ATP (Fig. 2*C*). NR_{II} also catalyzes the isotopic exchange between [^3H]ADP and ATP (data not shown). We could not detect any ATPase activity in NR_{II} (data not shown). It is therefore likely that the NR_{II}-phosphate formed in the reversible reaction of NR_{II} and ATP is an essential intermediate in the transfer of the γ-phosphate of ATP to NR_I.

The Phosphate in NR_{II}-Phosphate Is Bound to Histidine. We determined the stability of NR_{II}-phosphate as a function of pH. As shown in Fig. 3, the phosphate is rapidly lost at a pH lower than 3 but is stable at pH 14. This acid lability and alkali stability is characteristic of N-linked phosphates (5, 12, 13); accordingly, we hydrolyzed ^{32}P-labeled NR_{II}-phosphate with 3 M KOH at 120°C to a mixture of amino acids and analyzed the products by two-dimensional chromatography (10). As shown in Fig. 4, the radioactive compound present in this mixture comigrated with synthetic τ-phosphohistidine. Phospholysine and phosphoarginine are well separated from phosphohistidine by this method (10).

Phosphorylation of NR_I. In our study of NR_I phosphorylation we took advantage of the acid lability of NR_{II}-phosphate in contrast to the acid stability of NR_I-phosphate. Treatment of a reaction mixture containing the two phosphorylated proteins with trichloroacetic acid results in the removal of phosphate from NR_{II}-phosphate and the precipitation of phosphorylated NR_I. The inset in Fig. 5 shows the time course of incorporation of phosphate into NR_I. The reaction is very rapid when the concentration of NR_{II} is high and occurs in two phases. NR_I-phosphate reaches initially a high level and then falls to a steady-state level that is reached after at most 15 min. Addition of an excess of unlabeled ATP to a reaction mixture in which the level of NR_I-phosphate had reached the steady-state value resulted in the disappearance of the radioactivity from NR_I-phosphate. Apparently, NR_I-phosphate is unstable. This instability is illustrated in Fig. 2 *A* and *C*, where it is shown that the incubation of NR_{II}-phosphate with NR_I results in the transfer of the phosphate to NR_I followed by its release as inorganic phosphate. The amount of NR_I-phosphate increased with increasing NR_{II} concentration to a maximal steady-state value corresponding to 0.3 mol of phosphate per mol of NR_I monomer.

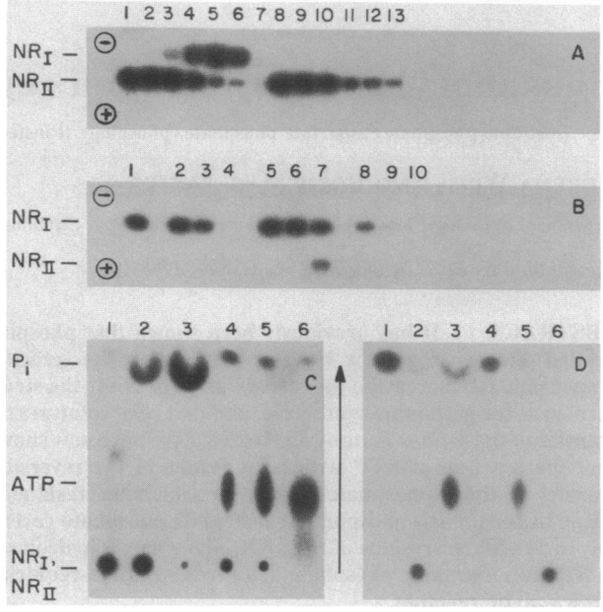

FIG. 2. (*A*) Transfer of phosphate from NR_{II}-phosphate to NR_I or ADP. ^{32}P-phosphorylated NR_{II} (360 nM) (track 1) and bovine serum albumin as a carrier (100 μg/ml) were incubated in 50 μl of 60 mM Tris·HCl/50 mM NaCl, pH 8, with 5 μM NR_I and 5 mM EDTA (tracks 2–4), with 5 μM NR_I and 5 mM $MgCl_2$ (tracks 5–7) or 2 mM ADP and 5 mM EDTA (tracks 8–10), or with 2 mM ADP and 5 mM $MgCl_2$ (tracks 11–13) at 37°C. After 1 min (tracks 2, 5, 8, and 11), 5 min (tracks 3, 6, 9, and 12) and 1 hr (tracks 4, 7, 10, and 13) 5-μl portions were added to 10 μl of a 0.5% $NaDodSO_4$ solution and analyzed by $NaDodSO_4$/PAGE and autoradiography. (*B*) Transfer of phosphate from NR_I-[^{32}P]phosphate to ADP. ^{32}P-phosphorylated NR_I (340 nM) and bovine serum albumin as a carrier (200 μg/ml) (track 1) were incubated in 60 mM Tris·HCl/300 mM NaCl, pH 8, with 2 mM ADP and 5 mM $MgCl_2$ (tracks 2–4), or 2 mM ADP, 5 mM EDTA, and 560 nM NR_{II} (tracks 5–7), or 2 mM ADP, 5 mM $MgCl_2$, and 560 nM NR_{II} (tracks 8–10) at 37°C. After 1 min (tracks 2, 5, and 8), 5 min (tracks 3, 6, and 9) and 1 hr (tracks 4, 7, and 10) 5-μl portions were added to 10 μl of a 0.5% $NaDodSO_4$ solution and analyzed by $NaDodSO_4$/PAGE and autoradiography. (*C*) Transfer of phosphate from NR_{II}-[^{32}P]phosphate to NR_I or to ADP. The reaction mixtures described in *A* were analyzed by thin-layer chromatography. After 1 hr of incubation at 37°C 2-μl aliquots were spotted on polyethyleneimine-cellulose thin-layer plates and chromatographed in 2 M LiCl/2 M HCOOH (1:1). The plates were autoradiographed. Track 1, NR_{II}-[^{32}P]phosphate (as *A*, track 1); track 2, NR_{II}-[^{32}P]phosphate, EDTA, and NR_I (as *A*, track 4); track 3, NR_{II}-[^{32}P]phosphate, $MgCl_2$, and NR_I (as *A*, track 7); track 4, NR_{II}-[^{32}P]phosphate, EDTA, and ADP (as *A*, track 10); track 5, NR_{II}-[^{32}P]phosphate, $MgCl_2$, and ADP (as *A*, track 13); track 6, [γ-^{32}P]ATP. (*D*) Transfer of phosphate from NR_I-[^{32}P]phosphate to ADP, analysis by TLC. The reaction mixtures described in *B* were analyzed by TLC as in *C*. Track 1, NR_I-[^{32}P]phosphate, ADP, and $MgCl_2$ (as *B*, track 4); track 2, NR_I-[^{32}P]phosphate, ADP, NR_{II}, and EDTA (as *B*, track 7); track 3, NR_I-[^{32}P]phosphate, ADP, NR_{II}, and $MgCl_2$ (as *B*, track 10); track 4, [^{32}P]P_i; track 5, [γ-^{32}P]ATP; track 6, NR_I-[^{32}P]phosphate. The arrow indicates the direction of chromatography.

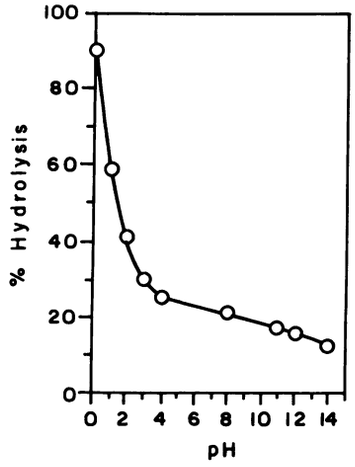

FIG. 3. Stability of NR_{II}-phosphate as a function of pH. ^{32}P-phosphorylated NR_{II} (225 nM) in 50 mM Tris·HCl, pH 8, containing bovine serum albumin at 100 μg/ml as a carrier, was added to the appropriate buffer solutions (pH 0, 1 M HCl; pH 1, 0.1 M HCl; pH 2, pH 3, pH 8, pH 11, pH 12: 1 M potassium phosphate; pH 14, 1 M potassium hydroxide; pH 4, 1 M sodium acetate) and incubated for 15 min at 37°C. Then 5-μl portions were spotted on nitrocellulose paper and washed with 45 ml of 50 mM Tris·HCl buffer, pH 8, and their radioactivities were measured in a Beckman liquid scintillation counter.

Instability of NR_I-Phosphate. We separated NR_I-phosphate from NR_{II}, NR_{II}-phosphate, and nucleotides by adsorption to heparin-Sepharose, from which it could be eluted by 1 M NaCl. The results illustrated in Fig. 6 show that incubation of NR_I-phosphate labeled with ^{32}P at 37°C in a solution containing $MgCl_2$ or $MnCl_2$ results in rapid loss of the phosphate group, which could not be prevented by adding an excess of EDTA. In every case the reaction followed first-order kinetics. Under these conditions the half-life of NR_I-phosphate is 3.5 min, irrespective of the initial NR_I-phosphate concentration. The NR_I-phosphate is considerably more stable when it is incubated in the absence of divalent cations: in this case, the half-life of NR_I-phosphate is approximately 1.8 hr. When NR_I-phosphate was denatured by treatment with 0.1% $NaDodSO_4$ (or 6 M urea, data not shown), its half-life increased to approximately 5.5 hr.

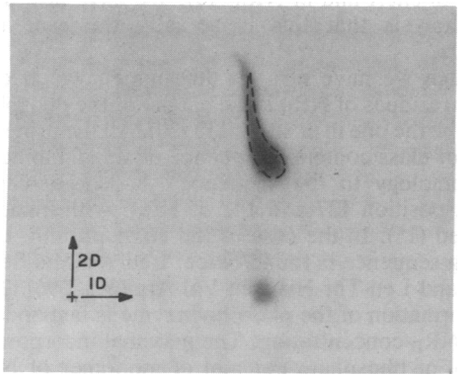

FIG. 4. Identification of [^{32}P]phosphohistidine after alkaline hydrolysis of NR_{II}-[^{32}P]phosphate. Phosphorylated NR_{II} (25 μg) was hydrolyzed in 3 M KOH (500 μl) for 100 min at 120°C in a sealed Eppendorf tube. The potassium ions were precipitated by the addition of 10% perchloric acid in the presence of a small amount of phenolphthalein as a pH indicator. The pH did not drop below 5. The supernatant was lyophilized, redissolved in 50 μl of water, and analyzed by two-dimensional chromatography on silica gel plates in the presence of phosphohistidine (10) [first dimension: *t*-butyl alcohol/methyl ethyl ketone/acetone/methanol/water/concentrated NH_4OH (10:20:20:540:5, vol/vol); second dimension: isopropyl alcohol/formic acid/water (20:1:5, vol/vol)]. The plate was treated with the Pauly diazo reagent and autoradiographed. The broken line indicates the position of phosphohistidine. Note that some NR_{II}-[^{32}P]phosphate is hydrolyzed to NR_{II} and inorganic phosphate after drying of the plates prior to chromatography in the second dimension.

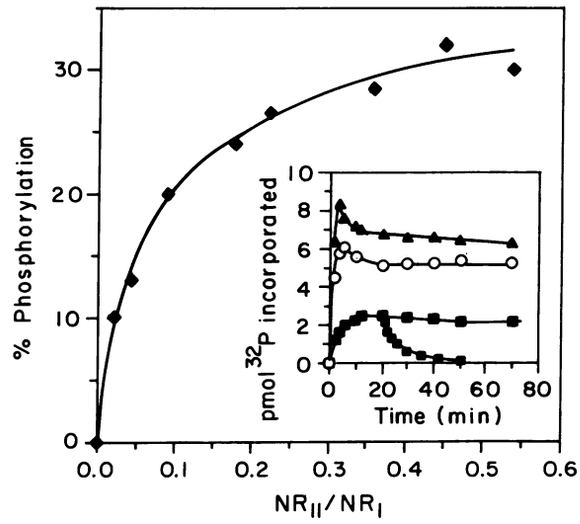

FIG. 5. Dependence of phosphorylation of NR_I on NR_{II} concentration. NR_I (2.75 μM) was incubated for 20 min at 37°C with [γ-^{32}P]ATP (0.4 mM, 1 Ci/mmol) and increasing amounts of NR_{II}. After 20 min, maximal phosphorylation was achieved. Portions (10 μl) were spotted onto glass filters and analyzed as described (2). (*Inset*) Phosphorylation of NR_I as a function of time at three different NR_{II} concentrations: ▲, 985 nM; ○, 490 nM; and ■, 60.4 nM. At the lowest NR_{II} concentration, excess unlabeled ATP was added at 20 min to give 20 mM; this produced the branch of the curve approaching 0 at 50 min.

The Phosphate in NR_I-Phosphate Is Bound to Aspartate. We examined the effects of exposing NR_I-phosphate to 0.1 M HCl, 0.25 M NaOH, or 0.8 M NH_2OH for 10 min at 37°C. Approximately 50% of NR_I-phosphate survived the treatment with acid, but only 20% survived the treatment with NH_2OH, and none survived the treatment with NaOH. These results rule out the possibility that the phosphate is bound to nitrogen or to a hydroxyl group, and they suggest that NR_I-phosphate is an acyl phosphate (12, 13).

This view was substantiated by the reduction of NR_I-phosphate with sodium boro[^3H]hydride, followed by the hydrolysis of the protein with 6 M HCl. This treatment should

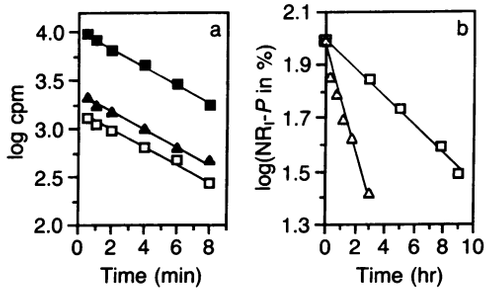

FIG. 6. Stability of NR_I-phosphate. ^{32}P-phosphorylated NR_I was incubated at 37°C under the conditions indicated below. (*a*) ^{32}P-phosphorylated NR_I (approximately 500 nM) was incubated in 25 mM Tris·HCl/500 mM NaCl, pH 8, containing 5 mM $MgCl_2$ (□), 5 mM $MnCl_2$ (▲), or 5 mM $MgCl_2$ and 20 mM EDTA (■). At the indicated time aliquots (8 μl) were analyzed by precipitation on glass filter paper with trichloroacetic acid as described (2). (*b*) ^{32}P-phosphorylated NR_I (NR_I-*P*), incubated in the same buffer, containing 20 mM EDTA (△) or 0.1% $NaDodSO_4$ (□), analyzed as in *a*.

result in the conversion of the acyl phosphate to δ-hydroxy-α-amino[^{3}H]valeric acid in the case of glutamyl phosphate or to [^{3}H]homoserine lactone in the case of aspartyl phosphate (14). We subjected the reduced and hydrolyzed NR_I-phosphate, as well as a reduced and hydrolyzed NR_I control to high-voltage paper electrophoresis in the presence of carrier unlabeled homoserine lactone and homoserine, and determined the radioactivity along the electropherogram. The results illustrated in Fig. 7*a* reveal a peak of radioactivity in the position corresponding to homoserine lactone that is not found in the control. The preceding large peak of radioactivity, also found in the control, presumably consists of β-alcohols generated by the reductive cleavage of peptide bonds, since the radioactivity in this peak was greatly reduced by treatment of the hydrolysate with periodate, a treatment that did not significantly affect the radioactivity of the peak in the position of homoserine lactone (data not shown) (14). The material in the homoserine lactone position in the periodate-treated material that had been subjected to electrophoresis was eluted and treated with alkali. Under these conditions homoserine lactone is converted to homoserine (14). The eluate, before and after treatment with alkali, was again subjected to high-voltage electrophoresis. As shown in Fig. 7*b*, the alkali treatment resulted in the disappearance of radioactivity from the position of homoserine lactone and its appearance in the position of homoserine.

These results show that the phosphate in NR_I-phosphate is attached to an aspartic residue. We can estimate from the ^{3}H radioactivity of the homoserine lactone in the electropherogram (Fig. 7*a*) and the ^{32}P radioactivity of NR_I-phosphate subjected to borohydride reduction and hydrolysis that we have accounted for a substantial fraction, approximately 15–18%, of the aspartyl phosphate of NR_I-phosphate.

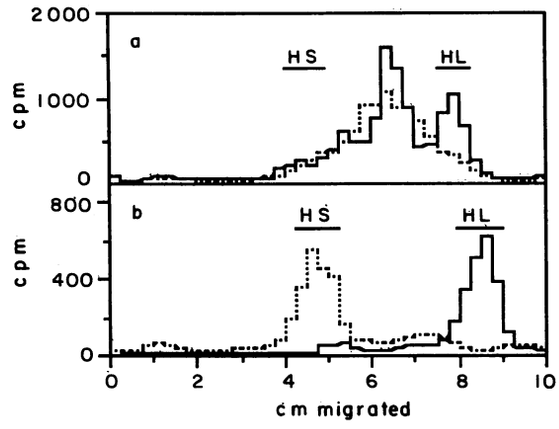

FIG. 7. High-voltage paper electrophoresis of acid hydrolysates of sodium boro[^{3}H]hydride-treated NR_I and NR_I-phosphate. Portions of 570 μg of NR_I-phosphate or 570 μg of NR_I were dissolved in 400 μl of dimethyl sulfoxide containing 5 mM boro[^{3}H]hydride (1 mCi) and incubated for 40 min at room temperature. The proteins were precipitated by addition of 4 ml of ice-cold 0.44 M perchloric acid. The precipitates were washed four times with 0.4 M perchloric acid, dissolved in 500 μl of 6 M HCl, and hydrolyzed in sealed tubes under nitrogen for 24 hr at 105°C. The hydrolysates were evaporated to dryness and washed four times with 500 μl of water to remove exchangeable tritium. Portions of the hydrolysates were analyzed by high-voltage paper electrophoresis in 7% formic acid, pH 1.9, in the presence of carrier homoserine (HS) and homoserine lactone (HL). The electropherograms were stained with ninhydrin (0.25% in acetone) and cut into 40 slices, and radioactivities were measured in liquid scintillation fluid (Aquasol). (*a*) Solid line, hydrolysate of NR_I-phosphate; broken line, control sample of reduced unphosphorylated NR_I. (*b*) The radioactive peak comigrating with homoserine lactone was extracted from the electropherogram overnight with 1 ml of 0.1 M HCl and evaporated to dryness. The eluted material was analyzed by high-voltage paper electrophoresis (solid line), and a portion was analyzed after treatment with 0.25 M NaOH for 72 hr at room temperature (broken line).

Reversibility of NR_I Phosphorylation. As shown in Fig. 2 *B* and *D*, the incubation of NR_I-phosphate with ADP results in the rapid disappearance of the phosphate group from NR_I-phosphate and its appearance in ATP. This reaction requires NR_{II} and Mg^{2+} and therefore constitutes a reversal of the NR_{II}-catalyzed transfer of the γ phosphate of ATP to NR_I.

DISCUSSION

In many instances the response of bacteria to environmental changes is mediated by the activities of a pair of proteins. Comparison of the amino acid compositions of these regulatory proteins has revealed the existence of two classes of proteins. The class with homology in the amino-terminal region consists of the effectors of the environmentally induced change. The other class, with homology in the carboxyl-terminal region, consists of the modulators of the activity of the effectors in response to the environmental signal (15). In the case of the regulation of the expression of genes in response to the availability of nitrogen, it was shown that the modulator, NR_{II}, the product of *glnL* (*ntrB*), catalyzes the phosphorylation of NR_I, the product of *glnG* (*ntrC*), and that NR_I-phosphate is responsible for the activation of transcription of nitrogen-regulated *glnA*, the structural gene for glutamine synthetase (2). This observation suggested the possibility that in all cases the activity of the effector is modulated by phosphorylation; and indeed, it has recently been shown that in the case of chemotaxis, the effector, the product of *cheY*, is phosphorylated by the modulator, the product of *cheA* (16–18). Moreover, it could be shown that the *cheA* product was capable of phosphorylating NR_I, and that NR_{II} was capable of phosphorylating the *cheY* product, though in each case the rate of the homologous interaction greatly exceeded that of the heterologous one (19).

The phosphoenzyme intermediate of *cheA* has been isolated (16, 17). Due to its stability at high pH and its lability at low pH, it was suggested that the phosphate is attached to histidine in *cheA* and in NR_{II} (19). We have now shown by direct analysis that this is actually the case in NR_{II}-phosphate.

Although we have not yet determined which of the 10 histidine residues of NR_{II} is the carrier of the phosphate, it is likely to be the one in position 139 (20). All the proteins of the modulator class contain a sequence of six amino acids, with great homology to the sequence Ala-Ala-His-Glu-Ile-Lys between position 137 and 142 of NR_{II}, with histidine fully conserved (15). In the case of the *cheA* protein, the corresponding sequence is found twice: Leu-Ala-His-Ser-Ile-Lys (46–51) and Leu-Thr-His-Leu-Val-Arg (391–396) (21).

The formation of the phosphoenzyme is fast and independent of NR_{II} concentration. The maximal incorporation was 0.35 mol of phosphate per mol of monomer of NR_{II}. It is possible that our NR_{II} preparation consists already in part of NR_{II}-phosphate.

The existence of a phosphoenzyme intermediate and the fact that it has been proven to be chemically competent in respect to the phosphorylation of ADP and of NR_I suggests that it is an essential intermediate for the phosphorylation reaction of NR_I and that the reaction follows a Ping-Pong mechanism.

The transfer of the phosphate of NR_{II}-phosphate to NR_I is rapid and requires Mg^{2+}. We have shown that NR_I is phosphorylated at an aspartic residue. The regulatory proteins of the effector class show great homology in their amino-terminal regions. Within the region of homology there are fully conserved aspartic residues in positions corresponding to 11 and 54 of NR_I, which are therefore good candidates for the phosphate carrier (15). It has been shown that the

phosphate is attached to one of approximately 110 amino acids that constitute the amino-terminal region of NR_I (22).

Acyl phosphates typically have a half-life of about 5 hr at pH 7 and 37°C (23), which is in good agreement with the half-life of denatured NR_I-phosphate (5.5 hr in 0.1% NaDodSO$_4$). Native NR_I-phosphate, however, is much more unstable, with a half-life of 1.8 hr in the absence of Mg^{2+} and about 3.5 min in the presence of Mg^{2+}. It appears, therefore, that the hydrolysis of the phosphate bond is an autocatalytic Mg^{2+}-dependent reaction. Our results are in good agreement with those of Keener and Kustu (22), who found a half-life of about 3.6–5.0 min in the presence of Mg^{2+} for native NR_I-phosphate (22).

It has previously been shown that the incubation of NR_I-phosphate with NR_{II} in the presence of P_{II} results in its dephosphorylation and in the loss of its ability to activate the initiation of transcription at *glnAp2* (2). These results appeared to indicate that NR_{II} combines with P_{II}, the product of *glnB*, to bring about the removal of phosphate from NR_I-phosphate. Our present results suggest the possibility that P_{II} merely blocks the phosphorylation of NR_I by NR_{II}-phosphate and that dephosphorylation of NR_I-phosphate reflects its intrinsic instability. However, that does not appear to be the case. We find the half-life of NR_I-phosphate to be 3.5 min, whereas in the earlier experiment NR_I-phosphate in the presence of NR_{II} and P_{II} lost more than half of its phosphate in approximately 1 min (2). This is in good agreement with Keener and Kustu (22), who found about the same half-life for the P_{II}/NR_{II}-catalyzed dephosphorylation reaction. They also found that ATP is required for this activity. Furthermore, experiments with intact cells have shown that mutants lacking NR_{II} are able to activate the expression of nitrogen-regulated genes, while mutants lacking uridylyltransferase are deficient in this ability (24). We assume that in mutants lacking NR_{II}, phosphorylation of NR_I by another member of the modulator class of regulatory proteins is responsible for the activation of the expression of nitrogen-regulated genes. In the mutant lacking uridylyltransferase, P_{II}, which cannot be converted to the innocuous P_{II}-UMP, combines with NR_{II} to remove the phosphate group of NR_I-phosphate regardless of the agent of phosphorylation.

This work was supported by Research Grants GM07446 from the National Institute of General Medical Sciences and AM13894 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, and by Grant DMB-8400291 from the National Science Foundation.

1. Hunt, T. P. & Magasanik, B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8453–8457.
2. Ninfa, A. J. & Magasanik, B. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5909–5913.
3. Ninfa, A. J., Brodsky, E. & Magasanik, B. (1988) in *DNA–Protein Interactions in Transcription*, UCLA Symposia on Molecular and Cellular Biology, ed. Gralla, J. (Liss, New York), in press.
4. Hultquist, D. E., Moyer, R. W. & Boyer, P. D. (1966) *Biochemistry* **5,** 322–331.
5. Hultquist, D. E. (1968) *Biochim. Biophys. Acta* **153,** 329–340.
6. Sheridan, R. C., McCullough, J. F. & Wakefield, Z. T. (1971) *Inorg. Synth.* **13,** 23–26.
7. Reitzer, L. J. & Magasanik, B. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5554–5558.
8. Ninfa, A. J., Ueno-Nishio, S., Hunt, T. P., Robustell, B. & Magasanik, B. (1986) *J. Bacteriol.* **168,** 1002–1004.
9. Nishigaki, I., Chen, F. T. & Hokin, L. E. (1974) *J. Biol. Chem.* **249,** 4911–4916.
10. Smith, R. A., Halpern, R. M., Bruegger, B. B., Dunlap, A. K. & Fricke, O. (1978) *Methods Cell Biol.* **19,** 153–159.
11. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
12. Fujitaki, J. M. & Smith, R. A. (1984) *Methods Enzymol.* **107,** 23–36.
13. Martensen, T. M. (1984) *Methods Enzymol.* **107,** 3–23.
14. Degani, C. & Boyer, P. D. (1973) *J. Biol. Chem.* **248,** 8222–8226.
15. Nixon, B. T., Ronson, C. W. & Ausubel, F. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7850–7854.
16. Hess, J. F., Oosawa, K., Matsumura, P. & Simon, M. I. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7609–7613.
17. Wylie, D., Stock, A. M., Wong, C.-Y. & Stock, J. B. (1988) *Biochem. Biophys. Res. Commun.* **151,** 891–896.
18. Hess, J. F., Oosawa, K., Kaplan, N. & Simon, M. I. (1988) *Cell* **53,** 79–87.
19. Ninfa, A. J., Ninfa, E. G., Lupas, A. N., Stock, A., Magasanik, B. & Stock, J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5492–5496.
20. Miranda-Rios, J., Sanchez-Pescador, R., Urdea, M. & Covarrubias, A. A. (1987) *Nucleic Acids Res.* **15,** 2757–2770.
21. Stock, A., Chen, T., Welsh, D. & Stock, J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1403–1407.
22. Keener, J. & Kustu, S. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4976–4980.
23. Post, R. L. & Kume, S. (1973) *J. Biol. Chem.* **248,** 6993–7000.
24. Bueno, R., Pahel, G. & Magasanik, B. (1985) *J. Bacteriol.* **164,** 816–822.